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A Decreased Production of IL12 In Vitro Is Associated With Isolation of Cytopathic HIV-1 Strains in HIV-1-Infected Patients

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The changes in type 1 (IL12, IFNγ, IL2) and type 2 (IL4, IL10) cytokine profiles may be associated with virological parameters of progression of the disease in HIV-1-infected patients. The production of cytokines was studied in LPS + PHA-activated whole-blood culture in HIV-1-infected individuals at different stages of the disease. The association was investigated between IL12p40 and IL12p70 profiles and other cytokines (IFNγ, IL4, IL10), as well as the isolation of cytopathogenic HIV-1 strains. The phenotype of HIV strains was studied by a micromethod based on P4 cell line, allowing detection of cytopathic effects of HIV-1 isolates (syncytium-induction and cell-killing without syncytium induction). The individual variations in IL12p40 and IL12p70 production were limited in the healthy controls. Low values were observed in HIV-1-infected patients. The production of IL12 (p40 and p70) and the IL12p70/IL4 ratio and the IFNγ/IL4 ratio were significantly lower in patients with cytopathic isolates compared with patients with noncytopathic isolates, and a correlation was obtained between the values of IL12 (IL12p40 and IL12p70) and those of IFNγ/IL4 ratio. There was no increase in the secretion of IL4 and IL10 in patients with cytopathic strains compared with other patients. The results indicate a decreased production of type 1 cytokines (IL12, IFNγ) in the presence of a relatively preserved production of type 2 cytokines (IL4, IL10) in HIV-1-infected patients. In conclusion, the defect of production of IL12 by whole blood is associated with virological correlates of progression of HIV-1 disease. ***J. Med. Virol. 55:209–214, 1998.***

KEY WORDS: cytokines; HIV-1-isolates; P4 cells

INTRODUCTION

Different mechanisms have been suggested to explain the pathogenesis of HIV-1 infection. It has been shown that interactions between viral and host factors take place in HIV-1 infection [Levy et al., 1993]. Several studies have outlined a correlation between the in vitro properties of HIV strains, such as rapid/high replication rate and ability to induce a cytopathic effect (syncytium-inducing variants), the rapid $CD4^+$ T-cell depletion and the progression of the disease [Connor et al., 1991; Koot et al., 1993]. Cytokine dysregulation induced by persistent HIV-1 infection has been postulated as being an important factor in contributing to the defective immune response of HIV individuals [Clerici et al., 1993]. In vitro the cytokine production shifts from a dominant type 1 (IL2, IFNγ) to a dominant type 2 (IL4, IL10) cytokine profile [Clerici et al., 1994]. It has been suggested that this change in cytokine profile is correlated with virological parameters of progression of the disease [Clerici et al., 1996; Vigano et al., 1996]. Together, these data indicate that virological and immunological parameters may be associated in advanced HIV-1 infection.

Recently, much attention has been given to the production of IL12 in HIV-1-infected patients. Interleukin-12 (IL12) is a heterodimeric cytokine of 70 kD formed by an H-chain (p40) and an L-chain (p35) encoded by separate genes [Kobayashi et al., 1989]. It has a variety of effects on T- and natural killer (NK) cells. IL12 is produced in response to bacteria, bacterial products, and intracellular parasites by cells with accessory or antigen-presenting ability [D'Andrea et al., 1992]. IL12 is a powerful inducer of type 1 immune responses and plays a central role in the resistance to intracellular

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pathogens [Trinchieri et al., 1994]. Researchers have reported that peripheral blood mononuclear cells (PBMCs) from HIV-1-infected patients produce lower levels of IL12p40 and p70 on stimulation, and Meyaard et al. [1997] reported similar results in a whole-blood culture system and the decreased IL12 production correlated significantly with decreased $CD4^+$ T-cell number in HIV-1 infected patients [Chehimi et al., 1994; Daftarian et al., 1995; Gazzinelli et al., 1995; Harrison and Levitz, 1996; Marshall et al., 1997]. These data suggest a defect in the production of IL12 for a type 1 immune response in HIV-1-infected patients.

IL12p40 and p70 production were studied in vitro with a whole-blood assay in HIV-1-infected individuals at different stages of the disease. The association was investigated between IL12p40 and p70 profiles, levels of type 1 (IFNγ) and type 2 (IL4, IL10) cytokines, and isolation of cytopathogenic HIV-1 strains.

PATIENTS AND METHODS

Patients

Fifty-seven HIV-1-infected individuals were obtained from the Department of Infectious Diseases, Tourcoing, France, and from the Department of Medicine, Valenciennes, France; their clinical status was defined according to the 1993 revised classification of the Centers for Disease Control (CDC) [CDC revised classification, 1993]. The HIV-1-infected individuals tested included 12 group A patients, 30 group B patients, and 15 group C patients. Thirteen healthy HIV-1 seronegative donors were also included as controls.

Blood Collection

Blood was drawn into sterile vacuum blood collection tubes (Vacutainer System, Becton Dickinson, Meylan, France), to which 20 U/ml of heparin (Panpharma, Fougère, France) was added. The blood samples were processed within 3 hours of collection.

Whole-Blood Culture

The tests were carried out in microtiter plates. After thorough mixing by repeated inversion of the tubes, 25 μl of heparinized whole blood were added to 225 μl of RPMI medium containing 25-μg LPS (LPS from *Salmonella enteritidis,* Sigma, St. Louis, MO) and 5-μg/ml PHA (PHA HA16 from Wellcome Diagnostics, UK). The cultures were incubated for 48 h at 37°C in a 5% CO_2 atmosphere, supernatants were then harvested and stored at –20°C until assayed for cytokines.

Cytokine Measurements

IL12p40, IL12p70, and IL10 measurements. IL12p40 and IL10 concentrations were determined by an enzyme-amplified sensitivity immunoassay (EASIA) kit (Medgenix Diagnostics, Fleurus, Belgium). IL12p70 levels were determined by an ELISA kit provided by Diaclone (Besançon, France).

IFNγ and IL4 measurements. IFNγ and IL4 measurements were obtained by using Screening Line EASIA (Medgenix Diagnostics). The assays used diluted purified anticytokine capture monoclonal antibody at 2.5 μg/ml in coating buffer, added at 200 μl/well to a protein-binding ELISA Nunc plate. The plates were covered and incubated for 4 hours at room temperature or overnight at 4°C, then washed 3 times and blocked with 0.5% bovine serum albumine (BSA) for 2 h at room temperature. After washing, the samples (200 μl) were added to the wells and incubated with diluted biotynilated anticytokine monoclonal antibody with phosphate buffer (1/10,000) for at least 2 h at room temperature with continuous shaking (700 rpm). After further washing, diluted streptavidin-peroxydase (Streptavidin-POD conjugate) in phosphate buffer solution was added at 1/10,000 for 30 min at room temperature with shaking (700 rpm). After washing, the wells were finally incubated with 200-μl tetramethylbenzadine (TMB) peroxidase substrate and the reaction stopped after 15 min by addition of 50 μl of stopping solution (H_2SO_4, 1.8 N). The plates were read at 450 nm and 492 nm (reference: 630 nm) by ELISA-Stat Fax 2100 microplates reader.

PBMC Cocultivation

1×10^6 freshly isolated PBMCs from HIV-1-infected patients were cocultured with 2×10^6 PHA-stimulated normal PBMCs; the coculture was performed in 1-ml RPMI medium supplemented with 10% fetal calf serum and human IL2 (final concentration: 5 U/ml) in 24-well plate. Half of the supernatants was harvested and replaced twice a week. Fresh activated cells were added once a week. The cultures were maintained for up to 4 weeks when possible.

The concentration of p24 HIV-1 antigen in the supernatants of PBMCs cocultures was assessed by using an HIV-1 p24 capture enzyme-linked immunosorbent assay (ELISA, Coulter Coultronis, France S.A., Margency, France). The presence of HIV-1 in the supernatants of PBMCs cocultures was detected in each patient tested.

Characterization of Cytopathic Effects of HIV-1 Isolates in P4 Cells

The P4 cells are HIV-infectible Hela-CD4 cells carrying the bacterial *LacZ* gene under control of the HIV-1 long terminal repeat (LTR), in which HIV-1 LTR-driven transcription of the *LacZ* gene and cytoplasmic accumulation of βgal are strictly dependent on the presence of the HIV transactivator Tat [Charneau et al., 1992].

The technique of detection of cytopathogenicity of HIV-1 isolates on P4 cells has been described in our previous work [Benyoucef et al., 1996]. Briefly: the P4 cells were resuspended in DMEM, 200 μl (2.5×10^4/well) were transferred to flat-bottom 96-well plates (Nunclon) and incubated overnight at 37°C. Then 10^5 cells from the coculture of PBMCs from patients were laid down to P4 cell cultures and the cultures were incubated for 3 days at 37°C. Every day, the P4 cells were observed under inverted microscope and the βgal

assay was performed on the third day of culture. The P4 cells were washed twice with PBS without Ca^{2+} and Mg^{2+}, fixed from 2 to 5 min in 0.5% glutaraldehyde, and incubated at 37°C for 6 h in the presence of 80 μl of reaction mixture. The reaction mixture was extemporaneously prepared as follows: 15 μl of X-gal (4-chloro-5-bromo-3-indolyl-β-galactoside) (Sigma, Milwaukee, WI), dissolved at 40 μg/ml in dimethylsulfoxide (DMSO), 30 μl of ferricyanide (200 mM), 30 μl of K ferrocyanide (200 mM), and 15-μl $MgCl_2$ (2M).

Statistical Analysis

The statistical significance of the differences of mean levels of cytokines in patients and controls was evaluated by the Student's test and correlations were made by use of simple regression tests.

RESULTS

IL12p40 and IL12p70 Levels Produced by Whole-Blood of Healthy Controls

The means ± S.D. of p40 and p70 determined in stimulated whole-blood cultures from 13 controls were 1306 ± 446 pg/ml and 65 ± 17 pg/ml, respectively. We studied the production of IL12p40 and IL12p70 in LPS + PHA-activated heparinized whole blood in five healthy individuals whose blood had been collected sequentially during a period of 5 months. The individual variations in IL12p40 ranged from 1% to 6%, and those in IL12p70 ranged from 2% to 12% (data not shown).

Levels of IL12p40 and IL12p70 Produced by Whole-Blood of HIV-1-Infected Patients

Individual IL12p40 and IL12p70 values produced in controls and HIV-1-infected patients are shown in Figure 1. The mean ± S.D. of IL12p40 values for group A, B, and C patients were 526 ± 533 pg/ml (P values vs. controls) ($P = 0.001$, n = 12), 400 ± 381 pg/ml ($P = 0.01$, n = 30), and 449 ± 251 pg/ml ($P = 0.0001$, n = 15), respectively. The mean ± S.D. of IL12p70 values for the same groups of individuals were 61 ± 63 pg/ml ($P > 0.5$), 41 ± 37 pg/ml ($P > 0.5$), and 12 ± 4 pg/ml ($P = 0.001$), respectively.

For IL12p70, a lower reference limit was set as equal to the mean - 2 S.D. of control group (31 pg/ml, n = 13). Low levels of IL12p70 were obtained in 5 out of 12 group A patients (40%) and 14 out of 30 group B patients (46%). It was noted that 16 HIV-1 patients showed normal production of IL12p40 but no detectable or at least a low level of IL12p70 (1 from 12 group A, 9 from 30 group B, and 6 from 15 group C patients).

Relationship Between the Levels of IL12 and Immunological and Virological Parameters of HIV-1 Infection

In 18 patients, the phenotype of HIV-1 isolates was studied. PBMCs from these patients were cocultured with PHA-activated normal PBMCs and the cytopathogenicity of isolates were studied in a P4 cell assay as described in Materials and Methods [Benyoucef et al., 1996]. A syncytium was not obtained at any time in the culture of PBMCs from nine patients; the phenotype of the strains of these patients was termed noncytopathic (NC). Syncytium formation was induced by isolates from three patients. The phenotype of these strains was termed syncytium-inducing (SI). PBMCs from six patients induced death of P4 cells without syncytium formation; the cytolysis of P4 cells appeared on the second day post-infection. The phenotype of the strains of these patients was termed cell-killing-inducing (CI) as described previously [Benyoucef et al., 1996]. Cytopathic effects in P4 cells appeared with cocultured PBMCs and with supernatants of cocultured PBMCs harvested as early as day 3 of coculture.

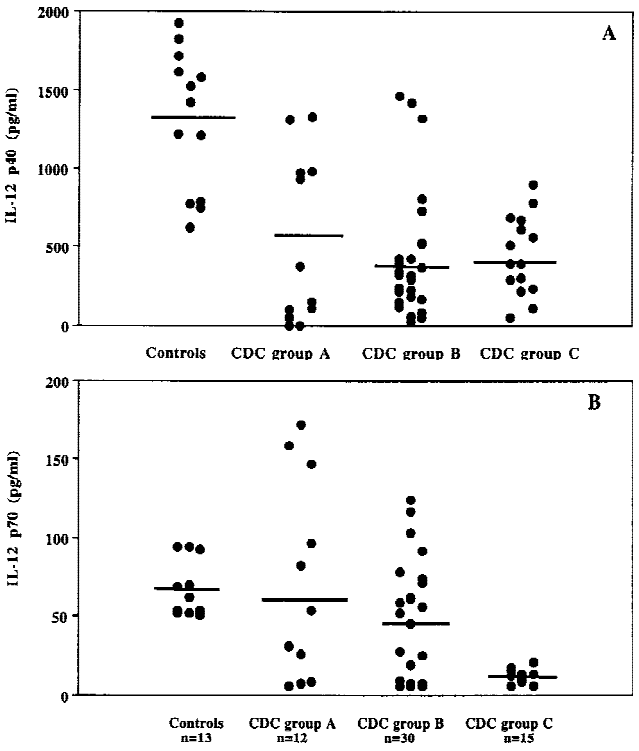

Fig. 1. Individual production of IL12p40 **(A)** and IL12p70 **(B)** in activated heparinized whole-blood culture from healthy HIV-1 seronegative controls (n = 13) and HIV-1-infected patients grouped according to the CDC classification as group A (n = 12), group B (n = 30), and group C (n = 15). Each symbol represents one subject. The horizontal bars represent the mean values.

In these patients and in seronegative controls, the production of IFNγ, IL4, and IL10 was studied in addition to IL12p40 and IL12p70 in the same samples of heparinized whole-blood cultures activated with LPS + PHA (see Table I). In healthy controls, the mean ± S.D. values of IFNγ, IL4, and IL10 were respectively 930 ± 156 pg/ml, 102 ± 54 pg/ml, and 527 ± 223 pg/ml (n = 13). IL12p40, IL12p70, IFNγ, and IL10 values in patients with cytopathic isolates (n = 9), including SI and CI strains, were significantly lower ($P < 0.01$) than the values in patients with noncytopathic isolates (n = 9) (see Table I). IL10 levels in patients were not higher than in controls. The concentrations of IL4 were not significantly different in patients with cytopathic isolates and in patients with noncytopathic isolates. In

TABLE I. Relationship between the cytopathic properties of HIV isolates and the profile of cytokine production in HIV-1 infected individuals*

Patient	CDC group	$CD4^+$ T-cell	Cytopathogenicity of HIV isolates	IL12p40	IL12p70	IFN_γ	IL_4	IL_{10}	$IL12_{p70}/IL_4$	$IFN_\gamma/IL4$
a	C1	475	NC	2670	172	621	3	940	57.3	207
b	B2	338	NC	1424	158	605	11	260	14.3	55
c	C2	259	NC	315	97	149	23	30	4.2	6.5
d	B2	465	NC	1394	147	510	6	510	24.5	85
e	B3	159	NC	315	92	528	5	220	18.4	105
f	B3	136	NC	1395	117	325	13	170	9	25
g	B3	162	NC	1461	124	473	5	470	24.8	94.6
h	C3	4	NC	192	21	192	8	45	2.6	24
i	C3	51	NC	213	74	341	6	110	12.3	56.8
Mean				1042	111	416	9	266	18.6	73
Standard deviation				843	46	172	6	284	16	60
j	C3	48	SI	81	6	46	21	32	0.3	2.2
k	B3	127	SI	376	45	30	23	90	2	1.3
l	C3	51	SI	25	31	25	18	80	1.7	1.4
m	C3	38	CI	143	25	69	10	40	2.5	6.9
n	C3	96	CI	187	31	150	26	90	1.2	5.7
o	C3	5	CI	82	6	102	20	80	0.3	5.1
p	C3	11	CI	114	21	187	4	50	5.2	46.7
q	B3	8	CI	60	6	96	5	80	1.2	19.2
r	B3	15	CI	54	6	35	15	24	0.4	2.3
Mean				124	20	82	15	62	1.6	10
Standard deviation				106	14	57	8	26	1.5	15
P value				0.01	<0.001	<0.001	0.14	0.049	0.011	<0.01

*Determination of cytopathic properties of HIV-1 isolates from peripheral blood mononuclear cells (PBMCs) of 18 infected patients and their clinical and immunological status. Cytokine production were measured in supernatants of whole-blood culture activated with LPS + PHA for 48 h. Measurements included IL12p40, IL12p70, IFNγ, IL4, and IL10 in pg/ml; IL12p70/IL4 and IFNγ/IL4 ratio values are presented. The mean ± S.D. values of each parameter obtained in cytopathic patients (n = 9) were compared to those obtained in noncytopathic patients (n = 9) by using the student's test and the *P* value of the test is presented.

these patients we obtained a correlation between IL12 (p40 and p70) and the following parameters: IFNγ ($P < 0.003$), IL10 ($P < 0.001$), IFNγ/IL4 ratio ($P < 0.001$), and $CD4^+$ T-cell number ($P < 0.001$), but there was no correlation between IL12 and IL4.

The IFNγ/IL4 ratio values and the IL12p70/IL4 ratio in patients with cytopathic isolates were significantly lower than the values in patients with noncytopathic isolates (see Table I). In contrast, the IFNγ/IL10 ratio, the IL12p40/IL4, and the IL12(p40, p70)/IL10 were not different in either group of patients (data not shown).

DISCUSSION

The current study of IL12 production in HIV-1-infected patients is different from those by other researchers since we studied the cytokine production in patients at different stages of the disease grouped according to the CDC classification. The nature of stimulus for IL12 production was different and we investigated the relationship between IL12 production and immunological and virological parameters of progression of HIV-1 infection. The ability of cells to produce cytokines in vitro is usually evidenced on isolated PBMCs stimulated with polyclonal activators; however, the isolation procedure may lead to cell activation, to a change in the ratio between the cells and thus depriving cells of important factors present in whole blood and reducing the reproducibility of cytokine production compared to whole-blood stimulation [Heumann et al., 1989; De Groote et al., 1992]. Therefore it was estimated that whole blood is the appropriate medium for studying cytokine production in vitro, in agreement with other authors [De Groote et al., 1992; Meyaard et al., 1997]. The in vitro stimulation of whole blood with PHA + LPS for 48 hours, used in the present study, was optimal for the various cytokines tested, as has been observed before [De Groote et al., 1992]. It may not be the stimulation most relevant to the in vivo situation; however, the value of nonspecific stimulation for studying the cytokine profile pattern in HIV-1-infected patients has been reported previously [Vigano et al., 1996; Meyaard et al., 1997].

It was shown that on stimulation with PHA + LPS, IL12p40 production in HIV-1-infected patients is lower than in controls at the different stages of the disease, whereas IL12p70 production was low in certain patients at the early stages of the disease (CDC group A and CDC group B) and significantly lower in more advanced patients (CDC group C). The HIV-1 infected patients had normal monocyte counts, therefore the impaired IL12 production could not be due to a reduced number of antigen-presenting cell, nor due to the inability of monocytes to produce cytokines since IL6 production was similar in the patients and in the healthy

controls (data not shown). A dissociation in the ability to produce IL12p40 and IL12p70 has been observed in certain patients, which agrees with data reported by Chehimi et al. [Chehimi et al., 1994]. The mechanism of this dissociation has not been elucidated yet, but may depend on different activation pathways for each molecule and may be related to the balance of various factors, such as cytokines acting independently as negative or positive regulators for IL12p40 and IL12p70.

Decreased production of IL12p70 in most of CDC group A and B patients was not obtained; this disagrees with the results of Chougnet et al. [1996], who observed a downregulation of IL12p70 in the early stages of the disease (Walter Reed classification, stage I-II). The discrepancy between the production of IL12 in HIV-1-infected patients in our work and in other studies may be due to the nature of the stimulus and the subsequent factors produced by different cell populations that are liable to affect the production of IL12p70. Indeed these authors used SAC or LPS for stimulation, whereas we used PHA + LPS for activation [Chehimi et al., 1994; Chougnet et al., 1996; Meyaard et al., 1997].

In agreement with Meyaard et al. [1997], we reported a correlation between IL12 production on stimulation with SAC and decreased $CD4^+$ T-cell number, whereas Chougnet et al. [1996] reported that impairment of IL12p70 production did not correlate with reduced $CD4^+$ T-cell number. The differences in the results of the previous reports could be related either to the technique used, since we stimulated with PHA + LPS, or to the clinical differences, especially the difference in $CD4^+$ T-cells in the peripheral blood between the series reported.

HIV-1 infection was suggested to be an immunovirological disease, therefore possible relationships was studied between immune response and biological properties of HIV-1 strains, especially the emergence of cytopathic isolates associated with progression to AIDS [Schuitemaker et al., 1992]. The microassay based on P4 cells used in this work to study the virological phenotype of HIV-1 allowed the detection of two types of morphological changes: one involved syncytium induction and the other cell-killing without syncytium formation, both related to the progression of the disease, whereas a microassay based on MT-2 cells allowed the detection of syncytium-inducing strains only (data not shown) [Benyoucef et al., in press]. For the first time to our knowledge we report that the production of IL12 (IL12p70 and IL12p40) is significantly lower in patients with cytopathic isolates compared with patients with noncytopathic isolates. The IFNγ/IL4 ratio and IL12p70/IL4 ratio values in patients with cytopathic isolates were significantly lower than the values in patients with noncytopathic isolates, but the values of other ratios, IFNγ/IL10, IL12p40/IL4, and IL12p70/IL10, were not different. In these patients a correlation was obtained between the values of IL12 (IL12p70 and IL12p40) and those of IFNγ/IL4 ratios. Together, the results are consistent with an association between a defective production of type 1 cytokines (IL12, IFNγ) and virological parameters in favor of a least favorable outcome of the disease, which agrees with the results of other authors [Clerici et al., 1996; Vigano et al., 1996]. However, in contrast with these studies, an increased secretion of IL4 and IL10 was not found in patients with cytopathic strains compared to patients with noncytopathic strains. The results are in agreement with a decreased production of type 1 cytokines in the presence of a relatively preserved production of type 2 cytokines, as has been reported previously [Meyaard et al., 1996].

In conclusion, the results are consistent with a defect of type 1 cytokines in HIV-1-infected patients with virological correlates of progression of the disease. The measurement of cytokines produced by heparinized whole blood on stimulation is simple and reproducible, and moreover only a small volume of blood is required. Therefore this approach is suitable for the investigation of large cohorts of patients in longitudinal studies in order to define the relationship between the disturbance of IL12 and other cytokines of type 1, and to determine whether an abnormal immune response with disturbance of cytokine production such as IL12 and IFNγ is responsible for the emergence of HIV-1 cytopathic phenotype or vice versa. The disarray of cytokine production obtained after nonspecific stimulation (PHA, LPS) should be representative of the potential secretion by mononuclear cells in patients. However, the role of cytokine production profile in response to HIV-1 antigens and the link with virological parameters of progression of the disease should be studied. Future research will be directed along this line in our laboratory.

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